# Utilization of Mieki as an Alternative to Yeast Extract in Batch Ethanol Fermentation

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# List of Abbreviation

| | |
|---|---|
| (v/v) | Concentration unit (volume/volume) |
| (v/w) | Concentration unit (volume/weight) |
| (w/w) | Concentration unit (weight/weight) |
| µL | Microlitre |
| CFU | Colony forming unit |
| CG | Commercial glucose / Dextrose |
| Conc. | Concentration |
| DCW | Dry cell weight |
| DNS | Dinitrosalicylic acid |
| EtOH | Ethanol |
| H | Fermentation time/Sampling (hours) |
| ME | Mieki/Ajieki |
| OD | Optical density unit |
| pH | Acidity, neutrality and alkalinity unit |
| Rpm | Revolution unit (rotation per minute) |
| Std | Standard |
| UV | Ultraviolet Ray |
| YE | Yeast extract |
| YM Media | Yeast and malt extract media |

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## ABSTRACT

In standard ethanol fermentation, yeast extract (YE) had been recognized as the most important component vital for microbial nitrogen supply, functions as growth promoter. However, yeast extract is very expensive to be applied in fermentation. Thus, a study was done with the aim in utilizing Mieki (ME) as an alternative compound to yeast extract for significant cost reduction especially in fermentation research field and ethanol production industry. Establishment of glucose standard curve was done with 30 replicates using DNS methods by Sumner, 1921 and Miller, 1959 which yield Y-value, 2.5353 and $r^2$-value, 0.9905. Batch ethanol fermentations were performed using 100g/L dextrose inoculated with 1% (v/v) *Saccharomyces cerevisiae* CSI-1 JCM15097 and proceeded for 24H. Two replicates of batch ethanol fermentation with 5g/L YE, four replicates of 2% (v/v) ME, and each for 4%, 8% and 10% (v/v) ME respectively. YE proved to show the best ethanol yield, 49.65g/L; followed by 2% (v/v) ME, 34.40g/L; and steady decline with the increase of concentration of Mieki to 10% (v/v) ME, 18.60g/L in 24H period. Performances of ME and YE for 36H onwards were comparable; 46.39g/L and 47.26g/L ethanol yield respectively.

Keywords: Mieki, Yeast Extract, *Saccharomyces cerevisiae* CSI-1 JCM 15097, DNS Method, Dextrose, Batch Ethanol Fermentation.

## *ABSTRAK*

*Ekstrak yis digunakan secara meluas dalam kebanyakan fermentasi etanol sebagai komponen yang penting dalam membekalkan nitrogen kepada mikrob, yang bertindak sebagai penggalak tumbesaran. Disebabkan kosnya yang tinggi, satu kajian telah dijalankan untuk mengkaji penggunaan Mieki sebagai bahan alternatif untuk menggantikan ekstrak yis di samping usaha untuk menggurangkan kos dalam fermentasi etanol terutamanya untuk bidang kajian dan industri penghasilan etanol. Penetapan piawai kecerunan glukosa dilakukan melalui 30 replikasi meliputi Kaedah DNS yang diperkenalkan oleh Sumner, 1921 dan Miller, 1959. Nilai Y dan $r^2$ yang diperolehi masing-masing adalah 2.5353 dan 0.9905. Fermentasi etanol kelompok dilakukan menggunakan 100g/L dekstrosa yang diinokulasikan dengan 1% (v/v) Saccharomyces cerevisiae CSI-1 JCM15097 dan diteruskan selama 24 jam. Dua replikasi dilakukan untuk fermentasi etanol kelompok dengan 5g/L ekstrak yis, empat replikasi untuk 2% (v/v) Mieki dan setiap satunya untuk 4%, 8% dan 10% (v/v) Mieki. Penggunaan ekstrak yis terbukti melalui penghasilan ethanol tertinggi, 49.65g/L; diikuti 2% (v/v) Mieki, 34.40g/L; dan penurunan sekata dengan peningkatan kepekatan Mieki kepada 10% (v/v) Mieki; 18.60g/L dalam tempoh 24 jam. Prestasi Mieki dan ekstrak yis untuk tempoh 36 jam ke atas adalah setara; penghasilan etanol masing-masing adalah 46.39g/L dan 47.26g/L.*

*Kata kunci: Mieki, Ekstrak yis, Saccharomyces cerevisiae CSI-1 JCM15097, Kaedah DNS, Dekstrosa, Fermentasi etanol kelompok.*

# CHAPTER 1

# INTRODUCTION

## 1.1 General Introduction

Ethanol fermentation industry commonly utilized yeast extract in their standard fermentation as nitrogen supply for bacterial growth promoter to maximize the level of fermentation efficiency (Bujang, 2008). Due to this application, bacterial growth is boosted to the maximum level both during inoculum preparation and fermentation by increasing the slope of the log phase. Thus, this obviously shortens the time taken for yeast to reach its maximum population number and subsequently be able to simultaneously produce its desired secondary metabolites, ethanol.

However, practicing the use of yeast extract is very expensive. High economic cost of fermentation mostly affects the research sector, where batch fermentations are done numerously as experimental replications and to test many variables with only limited amount of funds. In addition, this may contribute to problem in bigger scale industry that mass-produced ethanol since their products constitutes 57% to 78% of their cost hence making the product not competitive in the market as biofuel compared to conventional fossil fuels (Howell, 2005) (Pruszko, 2006).

To seek alternative to this problem, this study was done focusing in utilizing Mieki as an alternative compound of yeast extract. Mieki was reported to contain about 24g/L nitrogen hence this can be used as microbial nitrogen supply as by yeast extract (Nomura *et al.*, 1998). Thus, the cost demand for growth promoter can be reduced from 224 up to 417 times cheaper.

Performance comparison between yeast extract and Mieki was observed throughout the study. It was done by comparing biomass production, residual glucose concentration, ethanol yield and fermentation efficiency. Biomass production was measured using two distinct technique namely spectrophotometric turbidity determination and dry cell weight methods. Both residual glucose concentration and ethanol concentration was determined using OSI Biosensor$^{TM}$ device, ethanol yield and fermentation efficiency mathematically determined from it.

## 1.2 Objectives

This study was done to fulfil the objectives stated below:

- To study the effects of Mieki concentration of ethanol production by *Saccharomyces cerevisiae* CSI-1 JCM15097 in batch fermentation system with controlled pH.
- To obtain the optimum Mieki concentration for the highest ethanol production by *Saccharomyces cerevisiae* CSI-1 JCM15097 in batch fermentation system with controlled pH.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Commercial Glucose / Dextrose Monohydrate

Glucose, $C_6H_{12}O_6$ in its pure form, is a white crystalline substance, to which the name of dextrose is sometimes applied, the term glucose being reserved for the more or less pure commercial products, which may be solid or viscous (MIDA, 1970).

Dextrose monohydrate is purified and crystallized D-glucose and it contain one molecule of water of crystallization where the term 'monohydrate' is used and 'dextrose' stands for the dextrorotatory form of its molecule (Sorini Corp., 2003). It has been used widely in food applications especially in bakery products, biscuits, alcoholic beverage, canned products, confectionary, meat industry and frozen desserts.

According to Malaysian Industrial Development Authority, MIDA (1970), production of glucose is made by the prolonged action of dilute hydrochloric acid or sulphuric acid under pressure upon starch, and is stated to be a mixture of glucose with varying proportions of dextrin and maltose. MIDA (1970) divided these into three types of commercial products which are, liquid, solid and crystalline. The liquid form, termed Grade 1.430 containing 14 – 18% water, is incomplete hydrolyzed product of the same process that yields the other two forms; the solid form is obtained by a continuation of the process until the dextrin content is reduced to 5% followed by neutralization of the liquor, then continued concentration Grade 1.5 and cooling in moulds; the crystalline variety is prepared by the same general process (MIDA, 1970).

Table 2.1: General properties of dextrose monohydrate (Hongyu, 2005) (Sorini Corp., 2003)

| | | |
|---|---|---|
| Solubility[1] | | |
| i) | Water | High |
| ii) | Alcohol | Low |
| iii) | Ether, chloroform and other organic solvent | Insoluble |
| Specific rotation[1] | | 52.0 – 53.5° |
| Assay[2] | | 99.5% (min.) |
| Moisture content | | 9.0% (max.) |
| pH[2] (50% aqueous solution)<br>pH of water = 6.0 ±0.2 | | 5.0 – 7.0 |
| Chloride[1] | | 200ppm (max.) |
| Sulfate[1] | | 200ppm (max.) |
| Loss on drying[1] | | 9.0% (max.) |
| Iron[1] | | 20ppm (max.) |
| Arsenic[1] | | 2ppm (max.) |
| Sulfur Dioxide[2] | | 25ppm (max.) |

[1]Dextrose monohydrate description (Hongyu, 2005)
[2]Dextrose monohydrate product data sheet (Sorini Corp., 2003)

According to Sorini Corporations (2003), dextrose has high fermentability and can be added even to a low calorie beer for complete fermentation to alcohol. This is due to the absence of carbohydrate residue remains that may otherwise able to increase the calorie of the beverage. Thus, the use of dextrose monohydrate really benefits in the control of ethanol fermentation, ethanol yield and glucose residue compared to other form of carbohydrate that are not well-characterized.

## 2.2 Ethanol Fermentation

### 2.2.1 Batch Fermentation Mode

According to The American Heritage Science Dictionary (2005), fermentation is the process by which complex organic compounds, such as glucose, are broken down by the action of enzymes into simpler compounds without the use of oxygen. The term 'batch' means that feeding is only introduced once which is before the fermentation process started and microbes utilize this until its depletion.

Energy in the form of two ATP molecules is produced through fermentation process, and this energy is lesser compared to the aerobic process of cellular respiration. In many bacteria, fungi, protists, and animals cells - notably muscle cells in the body - fermentation produces lactic acid and lactate, carbon dioxide, and water. In yeast and most plant cells, fermentation produces ethyl alcohol, carbon dioxide, and water (The American Heritage Science Dictionary, 2005).

Batch fermentation is the most suitable feeding type for determination of parameter effects variables towards fermentation production. It required shorter time, lower cost and less technological knowledge than employment of continuous fermentation. However, batch fermentation also has its drawbacks. According to Dombek and Ingram (1987), the rate of ethanol production would only reach its maximum for a brief period of time early in the process and it declines rapidly as ethanol accumulates in the surrounding broth. But, for comparison and optimization analysis rather than production, mentioned effects do not bring much problem.

### 2.2.2 Ethanogenic yeast *Saccharomyces cerevisiae* CSI-1 JCM15097

According to Goffeau (1996), '*Saccharomyces'* derives from Latinized Greek and means 'sugar mold' or 'sugar fungus', *saccharo-* being the combining form sugar- and '*myces'* being 'fungus'. '*Cerevisiae'* comes from Latin and means 'of beer' (Goffeau, 1996).

All strains of *Saccharomyces cerevisiae* can grow aerobically on glucose, maltose, and trehalose and fail to grow on lactose and cellobiose. It was shown that galactose and fructose were two of the best fermenting sugars (Goffeau, 1996). The ability of yeasts to use different sugars can differ depending on whether they are grown aerobically or anaerobically.

All strains can utilize ammonia and urea as the sole nitrogen source, but cannot utilize nitrate since they lack the ability to reduce them to ammonium ions (Goffeau, 1996). Besides that, yeast can also utilize most amino acids, small peptides and nitrogen bases as a nitrogen source. Histidine, Glycine, Cystine and Lysine are however, not readily utilized (Goffeau, 1996). *S. cerevisiae* does not excrete proteases so extracellular protein cannot be metabolized (Goffeau, 1996).

Fermentation product, ethanol however may negatively affect the growth of yeast. It has been reported to damage mitochondrial DNA in yeast cells and to cause inactivation of some enzymes, such as hexokinase and dehydrogenase (Augustin *et al.,* 1965) (Ibeas and Jimenez, 1997) (Nagodawithana and Steinkraus, 1976). Nevertheless, some strains of the yeast *Saccharomyces cerevisiae* show tolerance and can adapt to high concentrations of ethanol (Ghareib *et al.,* 1988) (Alexandre *et al.,* 1994). Many studies have documented the alteration of cellular lipid composition in response to ethanol exposure (Beaven *et al.,* 1982) (Chi and Arneborg, 1999) (Ingram, 1976) (Kajiwara *et al.,* 1996) (Mishra and Prasad, 1989)

(Swan and Watson, 1999). It has been found that *S. cerevisiae* cells grown in the presence of ethanol appear to increase the amount of monounsaturated fatty acids in cellular lipids (Alexandre *et al.*, 1994) (Beaven *et al.*, 1982) (Sajbidor *et al.*, 1995). Since cell membranes have received extensive consideration as primary targets of ethanol stress, many reports have suggested a relationship between the fatty acid compositions of lipid membranes and ethanol stress tolerance (Alexandre *et al.*, 1994) (Beaven *et al.*, 1982) (Ghareib *et al.*, 1988) (Mishra and Prasad, 1989) (Sajbidor *et al.*, 1995). Although the correlation between ethanol tolerance and increased degree of fatty acid unsaturation of membrane lipids of *S. cerevisiae* is well documented, a causal relationship is not yet established.

Table 2.2: Taxonomic classification of *Saccharomyces cerevisiae* (Goffeau, 1996)

| | |
|---|---|
| Kingdom | Fungi |
| Phylum | Ascomycota |
| Class | Hemiascomycetes |
| Order | Saccharomycetales |
| Family | Saccharomycetaceae |
| Genus | *Saccharomyces* |
| Species | *Saccharomyces cerevisiae* |

## 2.3 Growth Promoter of Microbial Cell Growth

### 2.3.1 Yeast Extract for Ethanol Fermentation

Yeast extract consists or nitrogenous organics which contains vitamins. It is manufactured through the autolysis of *Saccharomyces cerevisiae* (Organotechnie, 2008). It is available in form of fine pale-yellow powder and easily soluble in water. Yeast extract has been use widely as a good supply of hydrosoluble B vitamins, free amino acids, peptides, purine and pyrimidine bases and growth factors that aid the propagation of bacteria that is essential in fermentation. Yeast extract is used as supply due to the fragility of vitamins because most of vitamins are heat-labile due to their organic structure (Bujang, 2008).

Yeast extract had been widely used and studied as nitrogen supply and growth factor not only for yeast but also other genus of microbes namely *Clostridium, Streptococcus* and *Monascus* (Leclerc *et al.*, 1998) (Pereira and Kilikian, 2007).

### 2.3.2 Mieki / Ajieki for Ethanol Fermentation

Mieki also known as Ajieki is a type of soy protein-acid hydrolyzate which contain high amount of protein and nitrogen content (Hipolito *et al.,* 2008). It is available in form of black liquid as a waste from by-products of Ajinomoto™ industries. Thus, it offers a very low and attractive economical cost for its utilization.

A study had been done utilizing Mieki as an alternative to yeast extract in year 2008 together with the installation, testing and training of a bench plant fermentor using the Ishizaki process for continuous bio-ethanol fermentation in Universiti Malaysia Sarawak. Ishizaki Process has been demonstrated to be able to maintain its consistency on commercial glucose and yeast extract (Hipolito *et al.,* 2008).

Mieki had also been proved suitable to be used as a replacement compound for yeast extract through the study of prepared aqueous culture medium for *Micrococcus percitreus* AJ 1065 utilizing 3ml Mieki and other components that was adjusted to pH 8.0 with KOH respective 45 liter batches of the aqueous medium were placed in 1750 litres fermentor (Konosuke *et al.,* 1976). The product from this study which was aromatic ethylamines can be recovered in pure state from the microorganisms, but cells and cell material are effective enzyme sources in the decarboxylation reaction (Konosuke *et al.,* 1976).

## 2.4 Post-fermentation Analysis

### 2.4.1 Spectrophotometric Turbidity Determination of Dry Cell Weight Estimation

Biomass can be predicted through determination of *S. cerevisiae* CSI-1 JCM15097 turbidity due to the nature of its cell structure. Spectrophotometric detection was done with visible and UV rays at 562nm where it detected yellowish white colour of the cell surface of yeast strain.

Spectrophotometric turbidity had been widely used especially in the determination of biomass for yeast cell suspension in inoculum prior to fermentation and enzyme assay (Washburne, 1996). This due to the advantage of the technique which are rapid, simple and does not damaged the yeast cell culture. Standard optical density curves are available for various microbes for cell forming unit and biomass determination (Forsburg, 1996). However, there is currently no standard optical density curve for *S. cerevisiae* CSI-1 JCM15097 biomass determination.

### 2.4.2 Dinitrosalicylic (DNS) Acid Methods (Sumner, 1921) (Miller, 1959)

Dinitrosalicylic acid was introduced by James B. Sumner in 1921 as a reagent for the estimation of sugar in normal and diabetic urine (Sumner, 1921). It has also been found to be a valuable reagent for the detection of albumin in urine and has been used for the determination of the titratable alkali of the blood (Sumner, 1923).

One of the chief errors in copper titration methods is the reoxidation of the cuprous oxide by atmospheric oxygen. With dinitrosalicylic acid, on the contrary, the amount of exposed surface has almost no effect, the same amount of reduction being given with tubes of all diameters. But with dinitrosalicylic acid the oxygen that is dissolved in the solution, while largely incapable of reoxidizing the reduction product, is able to destroy part of the sugar (Sumner, 1923).

It has been found that the addition to the reagent of a considerable amount of Rochelle salt largely prevents it from dissolving oxygen and greatly increases the amount of color given by small amounts of glucose. As used in the present modified manner, the amount of color given by 1mg of glucose is increased by about 65 per cent (Sumner, 1923).

As originally used, dinitrosalicylic acid gave practically no color with as little as 0.1mg of glucose, as nearly all of the glucose was oxidized by the dissolved oxygen. With the present modification the reaction is very dependable and the color values show such a good proportionality that the color given by 1mg of glucose can be compared without error with that given by either 0.5 or 2.0mg of glucose. Fructose and galactose have the same reducing power as glucose; 90mg of arabinose. Xylose is equivalent to 100mg of glucose; and 124mg of either anhydrous lactose or maltose are equivalent to 100mg of glucose (Sumner, 1923).

### 2.4.3 Ethanol and Glucose Determination using OSI™ Biosensor BF-5D

Oji Scientific Instruments from Japan had developed the biosensor which promptly measures the biochemical compound such as the saccharide and alcohol (OSI, 2009). Biosensor BF-5D was one of Biosensor series produced by the company. There were no much technical data available on how the equipment works and the concept on mechanism in it because the product was new in the market and its section in the company's website was still under construction.

In study done by Hipolito and his associates (2008), OSI™ Biosensor-BF-5D was used in the detection of glucose and ethanol concentration. Basically, Biosensor BF-5D externally appeared almost similar to HPLC equipment where it has one inlet for insertion of sample via syringe. At the initial state of its operation, Biosensor BF-5D would required 15-20 minutes for it to gain its operating temperature and able to process sample. Calibration must be done once prior to sample's quantification with glucose and ethanol standards supplied by the manufacturer. Detection and quantification of each sample's insertion required 60 seconds to complete and yield result.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Glucose Standard Curve Establishment

### 3.1.1 Preparation of Dinitrosalicylic Acid (DNS) Solution (Miller, 1959)

Chemicals required consisted of 1% (w/v) 3, 5-dinitrosalycylic acid, 0.2% (w/v) phenol crystal, 0.05% (w/v) sodium sulphite and 1% (w/v) were weighed. All except sodium sulphite were poured inside an aluminium foil-covered-beaker with addition of distilled water. It is then stirred using magnetic stirrer at 450rpm for 15 minutes in ambient temperature. Prior to use, the weighed sodium sulphite was added into the mixture with additional stirring for a short time. It was introduced later in the protocols to avoid the deterioration of the quality of the DNS solution due to lagging in time. Once mixed with sodium sulphite, DNS reagent would only yield valid result for the next 4 – 6 hours.

### 3.1.2 Sample Intuitive Dilution

Sample from glucose solution of concentration of 1g/L was subjected to several series of dilution. In the study, glucose with concentration of 1g/L was diluted into series of 0.1g/L, 0.2g/L, 0.3g/L, 0.4g/L and 0.5g/L concentration for each set of analysis.

### 3.1.3 DNS Solution-Sample Mixture and Reaction

DNS reaction was done by mixing 3mL sample with 3mL DNS solution in test tubes and incubating it in boiling water for 15 minutes for the redox reaction to be completed. The completeness of the reaction was marked by the change in colour of the solution.

### 3.1.4 Spectrophotometer Colorimetric Determination

After completion of the reaction, test tubes were cooled in a beaker of cold water followed by addition of 1mL Rochelle salt for colour stabilization preventing fluctuation during spectrophotometer reading. The solution mixture was then vortexed until homogeneous then poured into cuvettes. These cuvettes were then subjected into UV and visible rays at 575nm for colorimetric determination by spectrophotometer device.

## 3.2 Ethanol Batch Fermentation Trial and Experimental Run

### 3.2.1 Activation and Subculture of *S. cerevisiae* CSI-1 JCM15097

Activation and subculture media were prepared using YM media powder stored in dark bottle manufactured by Fluka, Sigma-Aldrich Corporation. Lab strain of yeast known as *Saccharomyces cerevisiae* CSI-1 JCM15097 was used in this study.

*Saccharomyces cerevisiae* CSI-1 JCM15097 was stored in the form of cell suspension with 20% (v/v) glycerol through employment of classical cryopreservation to preserve the cell structure, freezing the essential DNA molecules, dehydrating the intracellular and extracellular enzymes to deactivate them to ensure the viability of the microbial cell for theoretically unlimited period for use in further fermentation.

Glycerol was mixed together with the cell suspension to protect the cell from formation of intracellular water crystal that may rip the cell open inside out when stored under the ultra low temperature condition. Classical cryopreservation was implemented through the slow cooling of cell suspension by directly placed the microcentrifuge tubes inside polystyrene box in -80°C freezer, without the need of liquid nitrogen to snap froze it. Classical

cryopreservation was already sufficient for preservation of cell suspension compared to storage of highly sensitive enzymes and DNA which required modern cryopreservation.

Prepared stock culture of *Saccharomyces cerevisiae* CSI-1 JCM15097 was thawed to ambient temperature. Activation and subculture media were prepared together prior to activation of *S. cerevisiae* CSI-1 JCM15097. It was stirred and put in each individual universal bottle and then subjected to wet sterilization through the usage of autoclave. Once cold to touch, only then two 1.5mL microcentrifuge tubes of *S. cerevisiae* CSI-1 JCM15097 were placed out from the -80°C freezer and thawed to room temperature. The sequence and period were important to ensure that the cell suspension was not thawed and remain for too long in room temperature before it was activated as it may cause the stock culture to lose its efficiency and activity.

YM media with weight of 2.10g were used to produce 100mL of subculture medium which then distributed in 10mL to each of 10 universal bottles. From 1.5mL microcentrifuge tube which contains 1mL stock culture, 200µL yeast cell suspension was revived in 10mL YM media stored in universal bottle at 30°C for 24 hours. A total of 5 sets of 10mL YM media were used to activate the whole volume of the stock culture.

Activation period started when 200µL yeast strain cell suspension was aspirated into each universal bottle which contained the activation media. Aspiration was done inside laminar flow hood using sterile pipette tips with strict aseptic technique to avoid contamination and introduction of wild types of yeast strain which may pose risk of ‘killer activity’. Once the 5 universal bottles were sealed, it was then placed inside 30°C incubator for 24H allowing the yeast strain to adapt with the media and proliferate to achieve sufficient biomass. Subculture was done in order to replenish nutrient content in media allowing further proliferation and dilute the present biomass content.

Microbial cells with volumes of 200µL from the previous 5 sets of universal bottles were then transferred into 5 new sets of YM media. These subculture sets were put inside incubator at 30°C for another 18 hours.

### 3.2.2 Preparation of Fermentation Inoculum

Dextrose monohydrate was used as carbon source for the fermentation media in this study. It was available at the UNIMAS FRST Biochemistry Laboratory in the form of fine white powder packed in 25kg nylon bags, supplied by CornProducts$^{TM}$ (Batch no: 70173142001). Commercial glucose medium with concentration of 50g/L and 5g/L yeast extract were used for inoculum preparation. For subsequent experiment variables, the yeast extract was replaced with Mieki of 2%, 4%, 8% and 10% (v/v) concentration, respectively.

One of the best set of subculture yeast was selected using visual observation of its growth by comparing turbidity between them. Using aseptic technique, the set was then poured and cultured in a fresh inoculation medium composed of 50g/L glucose and 5g/L yeast extract with volume of 90mL. Inoculation media reached total volume of 100mL after addition of 10mL selected subculture set. This was then incubated at 30°C for 12 hours.

Inoculum period was marked by the transfer of total content of one universal bottle containing subculture yeast strain into inoculum flask containing specified fermentation medium. Inoculum phase was important as introduction of *S. cerevisiae* CSI-1 JCM15097 to fermentation media allowing it to adapt and proliferate in it. Besides that, it also takes part in the progression microbial growth phase of yeast strain covering the lag and some portion of the exponential phase ensuring that it simultaneously produce secondary metabolite product, ethanol, once inoculated inside fermentor for batch fermentation.

Inoculum medium was prepared to be in the same composition with the fermentation medium in the selected growth promoter type and concentration, but different dextrose concentration which was set to 50g/L instead of 100g/L in 1L fermentation medium. It was to prevent dextrose concentration variables to be accidentally introduced together into fermentation medium. Inoculum phase took 18H to complete, thus theoretically this was already sufficient for the yeast strain to complete its lag phase and utilize all the present sugar until completely depleted just prior to inoculation. Thus, the total concentration of fermentation medium set to 100g/L with the addition of inoculum medium.

### 3.2.3 Preparation of Fermentation medium and Biostat-B Biofermentor Device

Fermentation medium was prepared by diluting 100grams of dextrose monohydrate inside 900mL de-ionized water with specified type and concentration of nitrogen supply.

Prior to the starting of fermentation, the fermentation vessel with 900mL fermentation media in it with top cover intact, falcon tubes, syringes, all connection terminals and tubes sealed with aluminium foils were subjected to wet sterilization protocols utilizing autoclave to ensure no contamination of other microbial component. Sterilization was done promptly after preparation of fermentation media to avoid proliferation of contaminant inside it that may consume the media. Acid and base solutions, namely 1M $H_2SO_4$ and 2M NaOH sterilization were unnecessary since microbes could not grow in it.

Tubes connecting the acid and base solutions to the Biostat-B Fermentor's control processing unit and to the fermentation vessel, probes terminals, agitator motor, water supply inlet and outlet hoses were confirmed to be connected correctly to avoid complication and fluctuation to the fermentation process.